Study of gene transfection enhancement and parameters optimisation using electroporation microchip

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A design for improving gene transfection efficiency, which provides simple fabrication, low voltage and power consumptions, and easy implementation is developed; this design entails combining high and low electric fields, adjusting the voltage, using different numbers of pulses with varying durations, and different types of buffers with differing conductivities. The human embryonic kidney 293 (293T) cells were used. It was shown that a combination of two units of high electric field (1600 V/cm) for 0.6 ms and three units of low electric field (800 V/cm) for 1.2 ms is the optimum combination. This combination yielded a survival rate greater than 70% and a transfection rate of approximately 45%. The transfection rate of this combination was 80–100% greater than that obtained using two pulses, and the transfection rate obtained using two pulses was 40–50% greater than that obtained using single pulses. However, the survival rate decreases when the pulse duration is considerably long, even for low electric fields; therefore the number of pulses and their durations should be limited. The hypoosmolar buffer yielded the largest transfection rate. It was shown that the conductivity of the buffer solution is an important parameter, with the appropriate value ranging within 1.0–3.6 mS/cm. The transfection rate of the 293T cells using a cytoporation buffer could be enhanced by adding potassium ions in concentration lower than 2 μg/ml.

1. Introduction: Gene transfection is an important research topic in modern genetics and therapeutic fields. Electroporation is a type of gene transfection. In this process, cells are placed in an electric field, creating a series of small holes in the cell membrane. Material, such as DNA, is then delivered into these cells. By means of experiments, researchers have calculated the electric current and voltage information in single-cell electroporation [1]. Two or multiple pulse modes were applied to understand the cellular uptake of fluorescent dextrans [2, 3]. Irreversible electroporation can produce a possibly damaging thermal effect [4]. A multichannel electroporation chip was used to generate multiple electric fields in a single chip [5]. The ability of consecutive low-voltage pulses to induce apoptosis deserves credible attention [6]. Recently, chips fabricated by means of microelectromechanical systems have been utilised to enhance electroporation efficiencies. The enhanced green fluorescent protein (EGFP) gene was successfully transfected, but the death rate was not low [7]. The site-specific enhancement of gene transfection by using an attracting electric field has been reported, and two different human cell lines have been tested, yielding transfection rates within 13–36% and survival rates greater than 31% [8–10].

A study has demonstrated some parameters optimisation of an electroporation microchip system used for gene transfection using the Taguchi method [11]. A method of electrophoresis-assisted single-cell electroporation was introduced, and the delivery rate was enhanced by using electrophoresis; however, the number of transfected cells was limited since the single-cell electroporation technique was employed [12]. A combination of one short high pulse followed by a series of four long low pulses yielded optimal gene transfer efficiencies [13]. However, the duration of the four long low pulses can be considered fairly large, and some of the obtained results were not explained. Further, the electroporation occurred within a defined section of the microfluidic channel via geometric variation, and the maximum transfection rate (or yield) was 14.4% in the device having a single narrow section and 21.2% in the device having multiple narrow sections, and the survival rate was 30–80%. The advantages of this design were simplistic fabrication, the possibility of realise single-cell behaviour, and ease of integration [14–16]. However, the length and width directly influenced the ratio of the high and low electric fields. As the channel dimensions, the input voltage became considerably high.

On the macroscale, the distance between the electrodes and the volume of solution in the transfection instruments are larger than those of the chips within the same electric field, and the voltage and power consumption are also larger. On the microscale, extra particles and reagents are added [17], complex chips or control method, like multichannel [5], chemical vapour deposition and back-side etch [7] and sharply variable channel widths [14–16], are used in order to enhance the transfection efficiency. However, some of these techniques are expensive, difficult to fabricate and control, or high power consuming. Therefore, it has become imperative to develop a device that can offer simplicity in design, be cost-effective and easily yield increased transfection rates.

2. Material and methods

2.1. Principle of operation: The Nernst-Planck equation can be used to characterise the DNA-molecule cell-membrane transport as follows [18–20]

$$\frac{\mathrm{d}C(t)}{\mathrm{d}t}\frac{V}{A_{\mathrm{P}}(t)} = -D\frac{qF}{RT}C(t)E - D\frac{\mathrm{d}C(t)}{\mathrm{d}x} \tag{1}$$

where $C(t)$ is the molar concentration of the DNA passing through the surface area $A_{\mathrm{P}}(t)$ of the permeable structure defects, V is the volume of the cell, q is the electric charge of the DNA, E is the electric field acting on the molecules, F is the Faraday constant, R is the gas constant, T is the absolute temperature and D is the diffusion constant.

2.2. Chip design and fabrication: The main parts in chip design are the electrodes and reacting chambers. An interdigitated structure was used in the electrode design, as it can create larger electric fields in electroporation experiments (Figs. 1*a* and *b*). When

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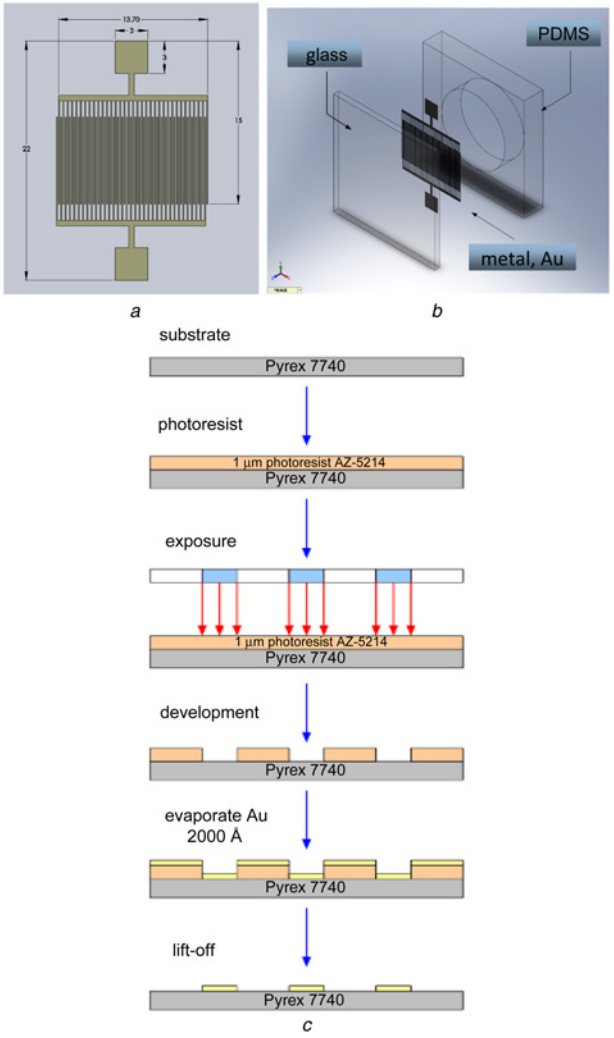

Figure 1 *Schematic design of whole electrode, exploded view drawing of chip, and fabricated processes of electroporation chip*
a Whole electrode (unit: mm)
b Exploded view drawing of chip
c Fabricated processes of electroporation chip

designing the chip, the usable cell size needs to be estimated initially. The distance between the positive and negative electrodes was maintained at 80 μm; the width of the electrodes was 120 μm. Four-millimetre-thick polydimethylsiloxane (PDMS) with a 5 mm-diameter opening was bonded to the glass surface with patterned electrodes to form the chamber. By using honeycomb-interlaced electrode structures, lower voltages could be obtained under the same electric field conditions.

A 4-inch glass wafer (Pyrex 7740, Dow Corning, USA) was used as the substrate, and AZ 5214 was used as the photoresist. Firstly, the photoresist was spin-coated on the substrate by means of a spin-coater (PM490, SWIENCO, Taiwan) at 3000 rpm for 30 s at 20°C (Fig. 1*c*). Secondly, the electrodes were patterned using exposure and development. Thirdly, an E-beam machine (CRTM-6000, ULVAC, Japan) was used to evaporate 2000 Å of gold on the substrate at the rate of 1.1–1.3 Å/s. Then, the lift-off process removed the unwanted parts. The conducting wire was then fixed and connected to the electrodes. PDMS was used to mould the reacting chamber of the chip. Finally, the sections of the electrode and reacting chamber were integrated to form the overall chip.

2.3. Preparation of biological samples: Human embryonic kidney 293 (293T) cells – easy to grow and transfect – have been widely

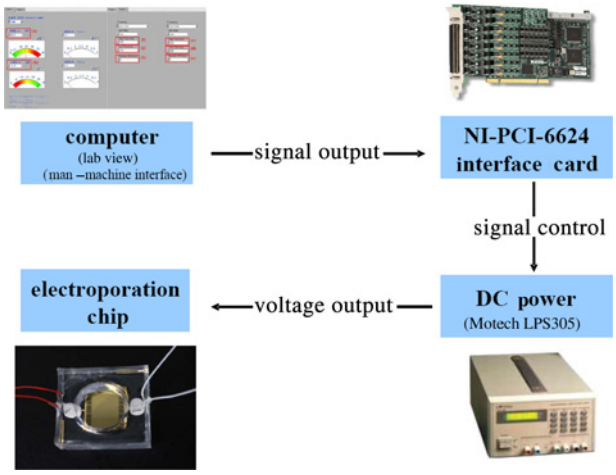

Figure 2 *Schematic diagram of cell transfection control system*

used in cell-biology research. In this study, 293T cells were used in the electroporation experiments, and the pYAO-2 DNA was used as the transfected gene. The pYAO-2 DNA consisted of the EGFP gene (Clontech, USA) driven by the cytomegalovirus promoter based on the plasmids pcDNA3.1-Hyg (Invitrogen, USA). A total volume of 100 μl of the cells at a concentration of 10^6 cells/ml was added prior to electroporation. Aliquots of the cells were mixed with aliquots (5 μg) of the plasmids and incubated for 10 min at room temperature. Then, the cells were subsided on the chip and the DNA was electroporated into the 293T cells. The cells were cultured in 24-well plates for two days. The EGFP was then expressed in the transfected cells. A green fluorescent protein was generated, which could be excited by UV light. The EGFP fluorescence in the transfected 293T cells was observed by using a fluorescence microscope (D1, Discovery, China), allowing its measurement in single cells. The transfection rates (%) were determined from the EGFP-positive cells/surviving cells. After the treatment with electroporation for two days, the 293T cells were collected and stained with 0.5% trypan blue in phosphate buffered saline. The stained cells were observed under a microscope (CKX41, Olympus, Japan). The surviving cells had not become stained. The cellular survival rates (%) could be obtained from the ratio of the stained non-blue cells to the total number of cells in the starting sample.

2.4. Experimental setup: In addition to the electroporation chip, the gene transfection experimental setup included a programmable control interface (LabVIEW) for controlling the time and frequency of the voltage. The power supply (LPS305, Motech, Taiwan) and an RS-232 port formed the other parts of the experimental setup. This setup could produce a maximum/minimum output voltage of ±30 V. We used the PCI-6624 waveform generation card (National Instruments, USA) controlled by the LabVIEW program, allowing adjustments of the experimental parameters. The gene transfection control system (Fig. 2) consisted of the electroporation chip, DC power supplier, a programmable controller (NI 9211 DAQ card) and a computer.

3. Results and discussion

3.1. Electric field test: After electroporation, the 293T cells were cultured for 24 h, and the pYAO-2 DNA fluorescence protein was created. When the protein was stimulated by UV light, the fluorescent 293T cells became visible, thereby achieving the goal. Each set of experimental conditions was repeated at least five times, with an average error of less than 15%. The transfection was tested for various electric fields (1200–2000 V/cm) by using the hypoosmolar buffer (Eppendorf, Germany), and the duration

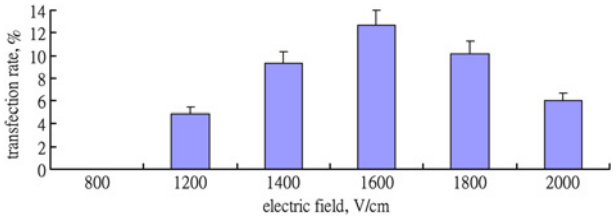

Figure 3 *Transfection rates using one pulse at various high electric fields at 0.4 ms and without low electric field by using hypoosmolar buffer*

of voltage input was 0.4 ms (Fig. 3). There was no transfection at 800 V/cm; consequently, the threshold value was greater than 800 V/cm. The transfection rate of 12.7% at 1600 V/cm was the largest; therefore 1600 V/cm can be considered as the optimum electric field for the transfection of 293T cells.

3.2. Test involving the number of pulses and duration: The number of pulses in the voltage input is an important parameter in the electroporation process. The results for different numbers of pulses (Fig. 4*a*) of the voltage input is shown in Fig. 4*b*, it can be seen that the transfection rate of 18.4% for two pulses is the highest. If the number of pulses is greater than two, the transfection rate decreases with an increase in the number of pulses. In the experiments, the duration of electroporation was also related to the transfection efficiency. Next, the duration of the pulses was tested. The duration between the high electric fields was 0.5 ms; various pulse durations (Fig. 5*a*) were compared, and the results are shown in Fig. 5*b*. The transfection rate of 22.8% for 0.6 ms pulses is the largest. If the duration of the pulse is larger than 0.6 ms, the transfection rate decreases as the duration of the pulse increases.

Since consecutive low voltage pulses can still induce apoptosis [6], various numbers of short low electric field pulses (1.2 ms) and one long low electric field pulse (80 ms) were selected, as shown in Fig. 6*a*. The hypoosmolar buffer was used, and the result is shown in Fig. 6*b*. The transfection rate of 45.1% obtained

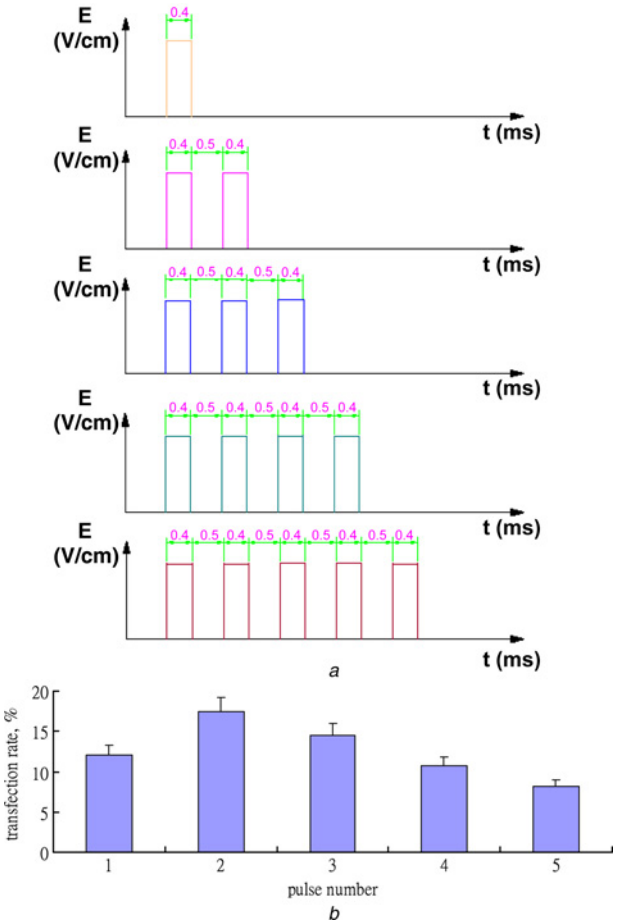

Figure 4 *Time distributions and transfection rates of various pulse numbers at 1600 V/cm*
a Time distributions
b Transfection rates

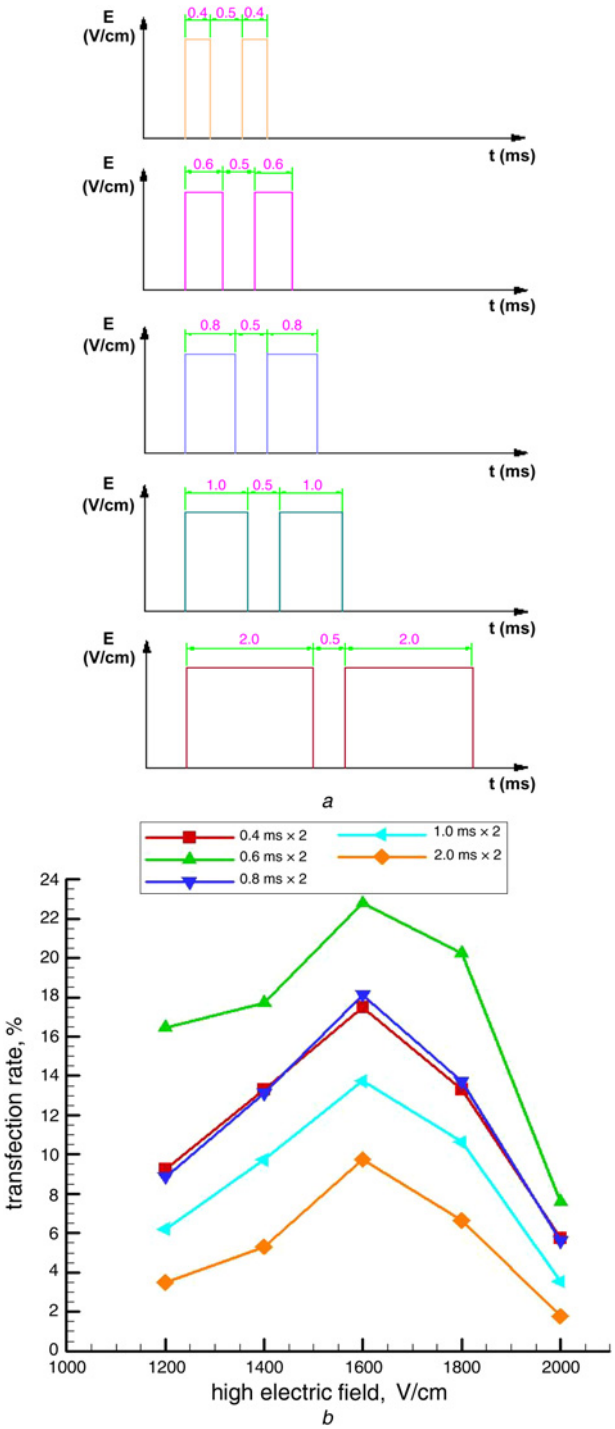

Figure 5 *Time distributions and transfection rates for various pulse durations of two pulses*
a Time distributions
b Transfection rates

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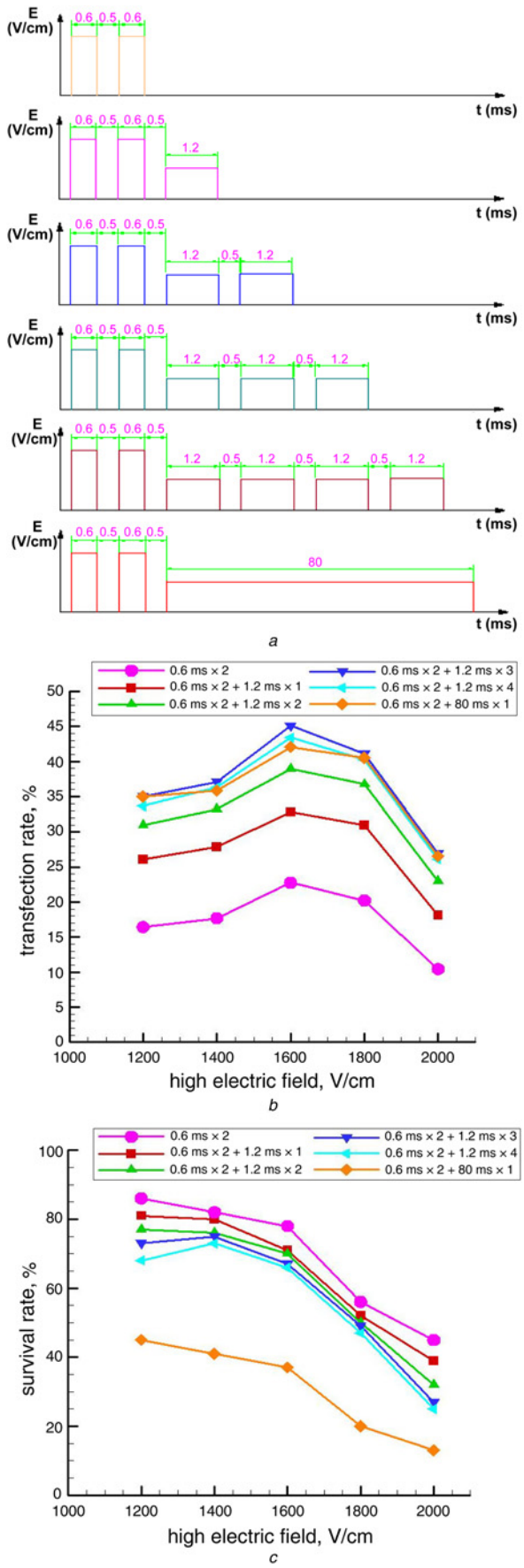

Figure 6 *Time distributions, transfection rates, and survival rates for various low electric field cases*
a Time distributions
b Transfection rates
c Survival rates

using the combination of 0.6 ms × 2 (E_H) + 1.2 ms × 3 (E_L) was the largest, and the transfection rate obtained using the combination of 0.6 ms × 2 (E_H) + 80 ms × 1 (E_L) was smaller, but the difference was not large. Therefore the efficiency of long low electric fields was not large. The transfection rates of 293T cells with high and low electric fields were 1.4–2.2 times larger than those with only high electric field for the various cases, as shown in Fig. 6*b*; therefore the introduction of high and low electric fields can indeed enhance the transfection efficiency. The efficiency of long low electric pulses was not necessarily higher than that of multiple short low electric pulses, but the survival rate obtained from the long low electric pulse was apparently lower, as shown in Fig. 6*c*; this can be attributed to apoptosis. Therefore the composition containing two units of high electric fields (1600 V/cm) of 0.6 ms and three units of low electric fields (800 V/cm) of 1.2 ms can be considered as the optimum configuration.

Next, the results obtained when using different numbers of pulses (2–8) of electric fields (1200–1600 V/cm) were compared. The transfection rate, as shown in Fig. 7, for two units of pulses of the electric field at 1600 V/cm was the highest. The transfection rate decreased with an increase in the number of pulses for the same electric field; the survival rate also decreased with an increase in the number of pulses. This implies that an increasing number of pulses might induce cell deaths, and the transfection rate also decreases when the number of pulses is greater than two. Therefore the number of pulses should be limited. Our results were different from the results obtained by Bureau *et al.* [13].

3.3. Buffer test: In the electroporation experiment, the cells and DNA samples were mixed in the buffer; therefore the buffer also plays an important role. Three buffers were selected: cytoporation (Cyto Pulse Sciences, USA), isoosmolar (Eppendorf, Germany) and hypoosmolar (Eppendorf, Germany). The transfection experiments using different high electric fields and buffers were conducted (Fig. 8). The transfection rate in the hypoosmolar buffer was the largest for different high electric fields; the maximum rate was 45.1% at $E_H = 1600$ V/cm. The transfection rate in the cytoporation buffer was the least. Next, the characteristics of these three buffers are discussed. Based on the technical specifications of the buffers, the conductivity of the cytoporation buffer is 0.08 mS/cm, and the conductivity of both

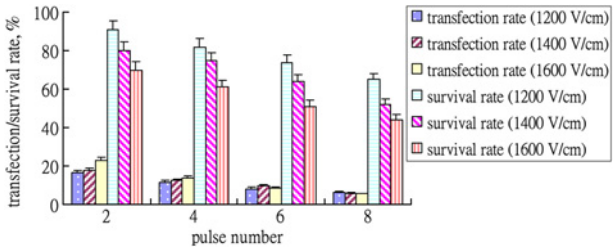

Figure 7 *Transfection rates and survival rates at various pulse numbers and high electric fields*

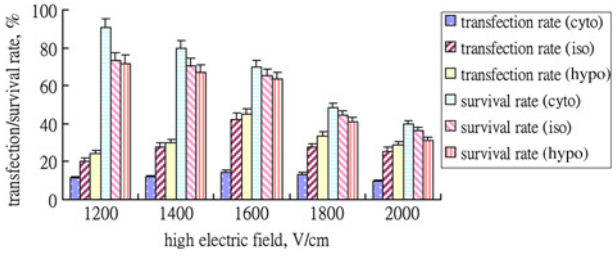

Figure 8 *Transfection rates and survival rates using 0.6 ms × 2 + 1.2 ms × 3 and at various buffers and high electric fields*

Table 1 Comparison of major ions concentration of three buffers

	K^+	Na^+	Ca^{+2}	Mg^{+2}
hypo	1055.2	0.529	0.130	0.002
iso	720.2	0.345	0.010	0.008
cyto	0.055	0.256	0.712	15.16

Unit: μg/ml

the hypoosmolar and isoosmolar buffers are the same, that is, 3.5 mS/cm at 25°C; therefore the conductivity might play an important role in the experimental results. It will be discussed in detail. We also compared the survival rates after electroporation using the combination of 0.6 ms × 2 + 1.2 ms × 3. The results show that the strength of the electric field influences the survival rate; when the strength of the electric field is larger, the survival rate is lower. When the electric field is smaller than 1600 V/cm, the survival rate is greater than 65%. When the electric field strengths are 1800 and 2000 V/cm, the survival rates are lower than 50 and 40%, respectively. By comparing the differences in the results obtained after using these three buffers, it is evident that the transfection rate was the largest when using the hypoosmolar buffer, but the survival rate was the lowest. The second highest transfection rate was obtained when using the isoosmolar buffer. The transfection rate was the lowest when using the cytoporation buffer, but the survival rate was the highest. The average survival rate determined using a transfection instrument (Multiporator, Eppendorf, Germany) when using the hypoosmolar buffer was approximately 57% at 1600 V/cm; therefore the survival rate resulting from the chip using high and low electric fields was higher than that obtained using the transfection instrument. This may be attributed to the damage resulting from the chip being lower because of the considerably less uniform electric field.

The conductivity difference between the cytoporation and hypoosmolar buffers was large, and the transfection rate of 293T cells using the cytoporation buffer was much lower than that using the hypoosmolar buffer. The potassium ion (K^+) concentration of various buffers was analysed by means of an atomic absorption spectrometer (SpectrAA 55, Varian, USA). The major ion concentrations in the composition of the three buffers are listed in Table 1. It is evident that the K^+ concentration of the cytoporation buffer was much lower. Based on the sodium–potassium-activated adenosine triphosphatase, or Na^+/K^+ pump, of the cells [21], it is now known that the carrier is an ATP-ase and that it pumps three Na^+ out of the cell for every two K^+ pumped in, as shown in Fig. 9. Therefore, it is possible to introduce K^+ to increase the

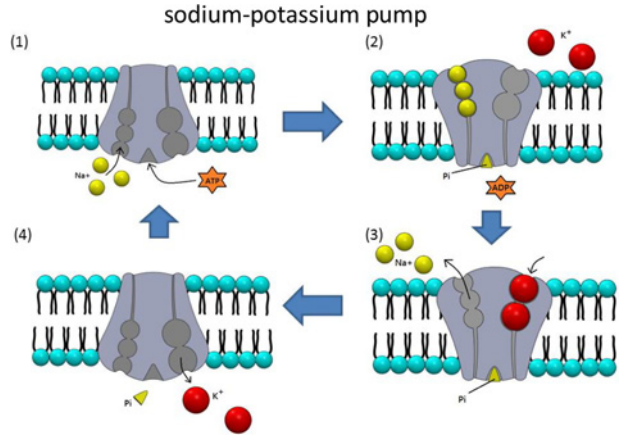

Figure 9 *Schematic drawing of sodium–potassium pump*

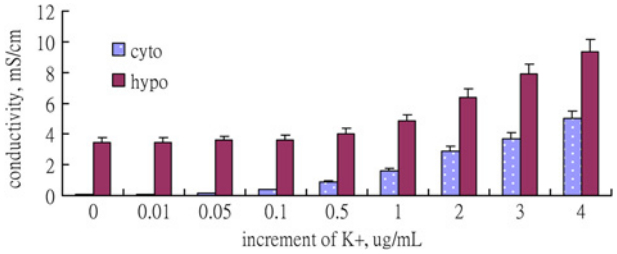

Figure 10 *Conductivity at various increments of K^+ in cytoporation and hypoosmolar buffers*

possibility of creating openings on the cell membrane, thereby enhancing the transfection rate.

We selected K^+ to adjust the conductivity. The conductivities of the mixtures of different buffers with K^+ were measured using a conductivity meter (SC-110, Suntex, Taiwan). The conductivities for various increments of K^+ in the cytoporation and hypoosmolar buffers are shown in Fig. 10. The conductivities increased with increasing K^+, and the conductivities of the K^+-hypo mixture was larger than those of K^+-cyto mixture for the same increment. The transfection results of 293T cells in the mixtures are shown in Fig. 11. It is evident that the transfection rate increased when the conductivity was increased in the K^+-cyto mixture at increments of $K^+ < 2$ μg/ml; the maximum transfection rate was 42.1% when the increment was 2 μg/ml. However, the transfection rate decreased when the conductivity was increased in the K^+-hypo mixture. The survival rates of the 293T cells in the mixtures are also shown in Fig. 11. The survival rate of 293T cells in the K^+-hypo mixture was lower than 40% for the increment of $K^+ >$ 0.5 μg/ml and the conductivity was larger than 4.2 mS/cm. Therefore, the conductivity of the buffer was also an important parameter, and the value of 1.0–3.6 mS/cm was considered better. The transfection rate of 293T cells using the cytoporation buffer could be enhanced by adding K^+ with the increment of K^+ maintained to be lower than 2 μg/ml. The transfection was tested for sodium ion (Na^+), magnesium ion (Mg^{+2}) and calcium ion (Ca^{+2}) in the cytoporation buffer. However, the transfection and survival rates at increments of Mg^{+2} and Ca^{+2} decreased. The transfection rate increased to 22% at the increment of $Na^+ = 1$ μg/ml, the survival rate decreased, and the increase of transfection rate was not apparent. It was found that the optimal electric field did not change with conductivity. These results can be used as a reference for the selection of buffers in the future.

3.4. Discussion: In the design, without microfabricated electrodes [5], the achieved transfection rate was 70%, and the survival rate was 80%; the efficiency of the system in [5] was better than that in the present study. However, the electric voltage used in the former study was in the range of 900–1500 V, which is significantly larger than that used in the present study (16 V). In studies using microfabricated electrodes [9], the achieved transfection rate was 35% and the survival rate was 40%; these

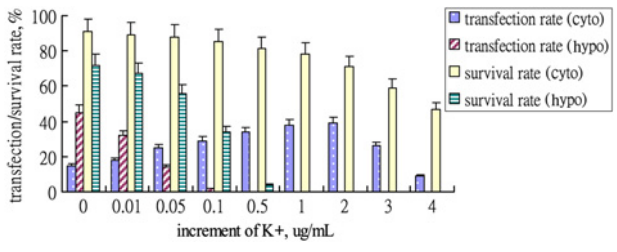

Figure 11 *Transfection rates and survival rates at various increments of K^+ in cytoporation and hypoosmolar buffers*

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values are lower than those of the chip system used in the present study. The high and low electric fields have also been generated by the geometric variations of the channel [14–16]. In these studies, the achieved transfection rate was 22% and the survival rate was lower than 60%. When the cells passed the regions of high and low electric fields in the channel, the cells stayed in the high electric field for almost 40–100 ms, which was considerably larger than that in the chip system used in the present study; the lower survival rate can be attributed to the foregoing. Besides, the power consumption of the design was considerably large; power consumption can be drastically reduced by using the chip designed in the present study. As the use of lower voltages of shorter durations, less harm may be caused to the cells; consequently, the survival rate obtained using the chip system proposed in the present study could be higher than that obtained using the system. In addition, the required amounts of cells and reagents are also considerably reduced because of a smaller reaction chamber, and no extra particles are needed.

4. Conclusions: In this study, we have developed a design for improving transfection, which involves simple fabrication, low voltage and power consumption, and easy implementation; this design requires changes only in the input voltage and requires no additional particles or reagents. It was tested and compared using various buffers and K^+ concentrations with respect to various parameters such as electric fields and the number of pulses and their durations. The experimental results have shown that high electric fields play the most crucial role in electroporation and the induction of high and low electric fields can indeed enhance the transfection efficiency. The transfection rate obtained when using a combination of two pulses was larger than that obtained when using single pulses; however, the number of pulses and their durations was still limited. The optimum combination of electric fields was $0.6\,\text{ms} \times 2$ (1600 V/cm) and $1.2\,\text{ms} \times 3$ (800 V/cm) when using 293T cells. The survival rates were higher than 70% with transfection rates of approximately 45%. The type of buffer and ion concentration were also important; the hypoosmolar buffer yielded the highest transfection rate. The conductivity of the buffer was also a major parameter, and the appropriate value ranged within 1.0–3.6 mS/cm. It was shown that the transfection rate of 293T cells using the cytoporation buffer could be increased by adding K^+ when the increment of K^+ was maintained to be lower than 2 µg/ml. The optimisation of the parameters of the electroporation microchip can provide additional transfection efficiency.

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